ever, drug lots or development procedures to give higher purity or yield would be most accurately compared by assay on one day with the same standard curves. Of course, the possibility of sample × assay × day interaction must not be ignored.

CONCLUSIONS

A screening procedure was used to evaluate the stability of a drug in the bulk form, and assays of streptovitacin A at 70° and room temperature for 60 days were compared. It is concluded that streptovitacin A is stable in the bulk form and is not subject to any thermal degradation which results in loss of biological potency.

The quantitative papergram assay against *S. pastorianus* for streptovitacin A is the method of choice rather than against *T. vaginalis*. The standard deviation, per cent of the mean, for the estimation of error of a single assay is 16% for the former, and 28% for the latter.

The variation of an assay within a single day is only one-half of the variation of that assay on various days. This phenomenon of daily variation has to do with the bias of the assay and not the day of sampling. Elimination of such daily assay variation could halve the variance.

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Selection, Evaluation, and Control of the Assay of the Pharmaceutical Product V

Importance of Assay Validity, a Case in Point

By EDWARD R. GARRETT†

Microbiological assays are used to measure drug potency but unwanted degradation products or impurities may positively interfere. A case in point is the tetracycline salts with normal moisture content in the cylinder-plate assay against *Bacillus subtilis*. Thermal degradation doubles the potency of tetracycline in this microbiological assay. Ultraviolet and infrared spectrophotometry indicate that aromatization and decarboxamidation are implicated. The resultant products have greater diffusivity and sufficient potency in the agar of the cylinder-plate assay method so that an *apparently* greater drug assay results. Dry tetracycline salts are thermally stable. Normal moisture content does not induce instability at usual storage temperatures. The kinetics of acid degradation of tetracycline phosphate and hydrochloride are the same.

The prediction of stability of drugs in pharmaceutical preparations (1) can be made only within the validity and reliability of the assay for drug. Previous papers in this series (2) have essentially considered the reliability or reproducibility of the assay.

The validity of an assay may be defined as its ability to measure that which it is supposed to measure. When biological assays are necessary, it is frequently assumed that degradation products of the drug do not demonstrate potency under the conditions of assay. This is not necessarily so. Biological assays are a complex sequence of physical, biological, and chemical effects. It is possible that the degradation products of a drug may not have the same pharmacological properties as the drug itself and yet give anomalous estimates of potency.

An interesting case in point is tetracycline and the assay in question is a classical microbiological method of antibiotic evaluation, the cylinder-plate assay (3) with *Bacillus subtilis* as the test organism.

EXPERIMENTAL

Biological Assay of Heat-Treated Tetracycline by Cylinder-Plate Assay.—Bulk tetracycline phosphate[1] with 2% moisture content was subjected to

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[1] Marketed by The Upjohn Co. as Panmycin Phosphate.

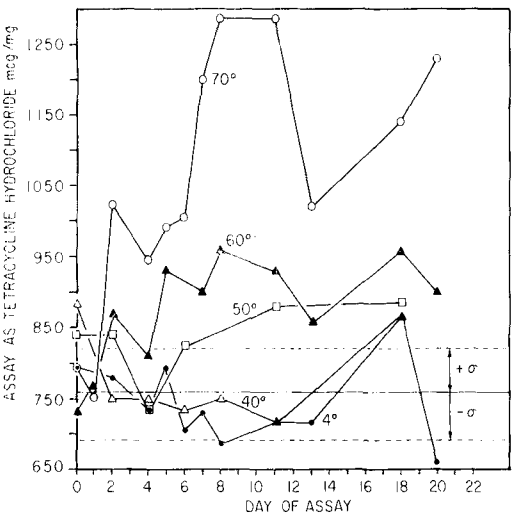

Fig. 1.—Cylinder-plate assay *vs. B. subtilis* of bulk tetracycline phosphate subjected to various temperatures. The solid line represents the mean of the 4° and 40° assays; the dashed lines represent the limits of the mean ± standard deviation.

70°, 60°, 50°, 40°, and 4° and assayed at intervals. The primary assay procedure was by the cylinder-plate method against *Bacillus subtilis* using tetracycline hydrochloride[2] as the standard. The collected assay data for the various temperatures are plotted as tetracycline hydrochloride against days of assay in Fig. 1.

Biological Assay of Heat-Treated Tetracycline Phosphate by Paper Chromatography.—Since the tetracycline phosphate was heat transformed to a material that had increased potency against *B. subtilis* in the cylinder-plate assay, it was deemed advisable to assay the material by paper chromatography against the same organism after the month of thermal treatment. The spot that moved like tetracycline was assayed as tetracycline against the standard. Table I gives the results.

TABLE I.—ASSAY AGAINST *B. Subtilis* OF PAPER CHROMATOGRAM OF TETRACYCLINE PHOSPHATE HEAT TREATED FOR ONE MONTH

Temp. of Treatment	mcg./mg. Assay A	mcg./mg. Assay B
70°	475	721
60°	767	900
50	958	925
40	829	1050
4°	925[a]	1050[a]

[a] This was the same material used as a standard in this assay.

Spectrophotometric Assay of Heat-Treated Tetracycline Phosphate.—The bulk tetracycline phosphate subjected to elevated temperatures was spectrophotometrically analyzed at pH values of 2.32, 4.88, and 9.25. Heat at 70° affected the spectra in that the apparent absorptivity at 218 mμ increased to a maximum of 45%, at 270 mμ to a maximum of 63%, and at 356 mμ decreased to 49.5% at pH 2.32.

[2] Marketed by The Upjohn Co. as Panmycin.

The absorbances at these various wavelengths and pH values became asymptotic with time at 70°. If the symbol for the asymptotic value at 70° is A_∞, then the first-order rate constants may be determined from the slope of plots based on the expression

$$\text{Log}\,(A_\infty - A) = -\text{kt}/2.303 + \text{constant}$$

Such plots for a given temperature were parallel, no matter the wavelength or the pH. Typical plots for 70°, 60°, 50°, and 40° treated material are given in Fig. 2. These were obtained at 218 mμ and at pH 2.32 in 0.005 *M* hydrochloric acid. The slopes of such plots are given in Table II.

TABLE II.—SLOPES OF POSSIBLE FIRST-ORDER HEAT DEGRADATION OF TETRACYCLINE PHOSPHATE

Temp.	Slope, $k/2.303$ in day^{-1}
70°	0.67
60°	0.0062
50°	0.0025[a]
40°	indeterminate

[a] Estimated but could be insignificant with respect to variability of the measurement.

Identification of Gaseous Product of Heat Degradation of Bulk Tetracycline Salts.—The bulk tetracycline phosphate darkened when subjected to temperature. The degree of darkening increased with temperature. The 70° material after a month's time was virtually dark brown. Within the bottles a gas pressure was generated. This gas was isolated and shown by infrared spectroscopy to be carbon dioxide.

Dried and normally hydrated tetracycline hydrochloride and phosphate were subjected to 70° temperature for several weeks. In *both* cases of hydration, decomposition to a gray-black mass was visually noted and CO_2 was determined to be present in both atmospheres. In both cases of the dried tetracycline, no discoloration was observed. It is thus concluded that CO_2 liberation and decomposition occur with moist tetracycline salt.

Solution Degradation of Tetracyclines.—Buffers were prepared of hydrochloric acid-potassium chloride for pH values of 1.2 and 2.2. The former was prepared with 250 ml. 0.2 *M* potassium chloride and

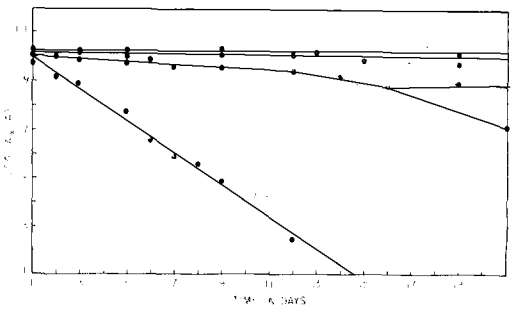

Fig. 2.—Apparent first-order rate plots for the thermal degradation of bulk tetracycline phosphate at various temperatures as based on ultraviolet spectrophotometry in pH 2 acid solution. The A_∞ is taken as the asymptotic absorbance with time of the 70° degraded material.

327.5 ml. 0.2 *M* hydrochloric acid and the latter with 250 ml. 0.2 *M* potassium chloride and 33.5 ml. 0.2 *M* hydrochloric acid (4). Both were brought up to volume in a 1-L. flask.

A 9.797 mg. quantity of tetracycline free base and 13.132 mg. tetracycline phosphate were each dissolved in 10 ml. dimethylformamide. Five milliliters of each solution were brought up to volume with 1.2 and 2.2 pH buffers, respectively. The resultant solutions were maintained at 30.3° and read at intervals on the Beckman model DU spectrophotometer at the maxima of tetracycline, 270 mμ and 356 mμ.

CALCULATIONS AND RESULTS

Anomalous Increase in Potency by Cylinder-Plate Assay of Heat-Degraded Tetracycline Salts.—The screening of bulk drug for instability can be readily effected by assaying heat-treated material at intervals of time. The assays of bulk tetracycline phosphate subjected to various temperatures are plotted as functions of time in Fig. 1.

Since the variation among assay values of the drug subjected to high temperatures was significantly outside the expected assay error, the mean and standard deviation (σ) in this figure is calculated solely from the 4° and 40° data.

The mean and standard deviation of the assay of this material as tetracycline were pooled from the entire month of study and are 760 and ±70 mcg./mg., respectively.

It is readily apparent that the material subjected to 70° and 60° significantly increased in apparent assay. The 70° material developed a potency of 1.7 times the mean value in 1.5 week's time. Although the increase in potency of the 50° material is not as statistically significant, it can be estimated as 1.15 the mean value. The 60° material developed a potency of 1.25 times the mean value.

There can be little doubt that the bulk tetracycline salt was transformed by heat to a new material that demonstrated increased potency *by this particular assay method.* These data do not permit any valid estimate of stability *as tetracycline,* since the assay method responds to degradation products other than tetracycline.

The ultraviolet spectrophotometric assay of the heat-degraded materials could provide a more quantitative estimate of rates of this transformation. Such estimates were given in Table II. Thus, if the ratio of rate constants at 10° intervals is chosen as $k_{70°}/k_{60°} \sim 100$ from Table II, the rate constant at 30° would be 6×10^{-9} day^{-1}. This would result in negligible spectrophotometric change in over a year at 30°. Even if the more disadvantageous ratio $k_{60°}/k_{50°} = 2.5$ were used, there would be negligible spectrophotometric change at 30° within a year.

The results of Table I confirm the spectrophotometric observations. The transformation of bulk tetracycline has a high thermal activation. Although the transformation has a high rate at 70°, it is much less at 60° and negligible at 50° and 40°. The best estimate from these data on the assumption that tetracycline is being assayed by this technique is that it is inherently stable in the bulk form at 30°.

Solution Degradation of Tetracycline Salts.—Plots of absorbance at 270 and 356 mμ, the maxima of tetracycline in acid media, against time in hours for tetracycline phosphate and hydrochloride at pH 2.2 and 1.2 are given in Fig. 3. The coincidence of data for phosphate and hydrochloride proves that both salt types undergo the same degradation at the same rate.

An example of the pseudo first-order plots of the absorbance data is given in Fig. 4 as based on the premise that

$$dx/dt = -k_H{}^+[H^+]x$$

where x is the concentration of tetracycline and $[H^+]$ is the hydrogen ion concentration. This integrates to

$$\log x/x_0 = -k_H{}^+[H^+]t/2.303 = -kt$$

where x_0 is the initial tetracycline concentration.

When absorbance is the measure of concentration, then

$$\log/A_t - A_\infty/ = -kt + \text{constant}$$

where A_t is the absorbance at time t and A_∞ is the asymptotic absorbance at infinite time and

$$k = k_H{}^+[H^+]/2.303$$

The rate of appearance of the chromophore at 270 mμ at pH 1.2 is 0.00211 and 0.00202 for hydrochloride and phosphate, respectively. The rate of disappearance of the chromophore at 356 mμ at pH 1.2 is 0.00200 for both.

The mean value of k in the fifth equation at pH 1.2 is thus 0.00203 and $k_H{}^+$ $[H^+] = 4.675\ 10^{-3}$ $hr.^{-1}$ Thus, $k_H{}^+ = 4.675.10^{-3}/10^{-1.2} = 7.41.10^{-2}$ 1/M/hr.

Thus, in general, the fraction of tetracycline remaining after acid degradation at a given pH and 30° may be calculated from the expression if specific acid catalysis is assumed

$$\log x/x_0 = -7.41.10^{-2}.10^{-pH}t/2.303 = 3.21.10^{-2}\,[H^+]t$$

It follows that a solution of tetracycline salt in 0.01 *M* hydrochloric acid undergoes 10% degrada-

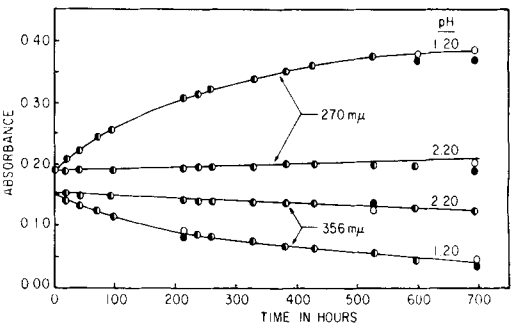

Fig. 3.—Change of absorbance with time for tetracycline hydrochloride, ⊙, and tetracycline phosphate, ●, at several acid pH values at 30.3°. The concentrations of tetracycline were 4.900 mg./L. of hydrochloric acid-potassium chloride buffer or 1.104×10^{-5} *M*. The coincident values are given as half solid circles, ◐.

TABLE III.—ULTRAVIOLET SPECTRAL DATA ON TETRACYCLINES IN ACID SOLUTION

Compound	λ	Absorptivity	λ	Absorptivity	λ	Absorptivity
Tetracycline hydrochloride, standard	218.5	29.23	270	39.52	356	29.85
Tetracycline phosphate, dried	217	26.03	269	33.82	356	25.31
Tetracycline phosphate, non-dried	218	23.49	270	30.74	356	27.34
Heat-degraded tetracycline phosphate, non-dried	218	34.0	270	50.0	356	13.5
Anhydrotetracycline	224	65.73	274	127.82	...	...

tion in 156 hours, 25% in 413 hours, and 50% in 990 hours at 30°. In one day, in 0.01 *M* hydrochloric acid there will be 1.8% decomposition and in 2 days, 3.5% decomposition. At 0°, on the assumption of a heat of activation of 15 Kg. cal./mol., approximately 1% decomposition will occur within a week's time at pH 2.0. The above information should be important in the keeping of standard solutions.

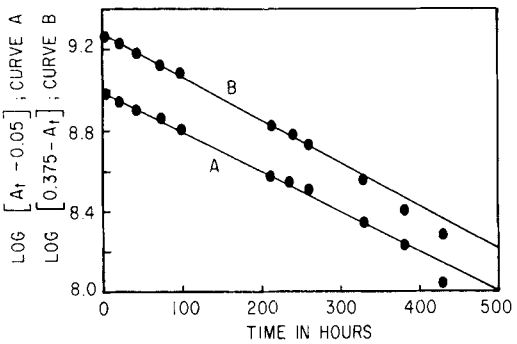

Fig. 4.—First-order rate plots for the acid catalyzed degradation of 1.104×10^{-5} *M* tetracycline hydrochloride in hydrochloric acid-potassium chloride buffer at pH 1.2 at 30°. Curve *A* represents the disappearance of the 356 mμ chromophore; curve *B* represents the appearance of the 270 mμ chromophore. The abscissa is the logarithm of difference of the asymptotic absorbance, A_∞, and the absorbance at any time, A_t.

DISCUSSION

The heat degradation of bulk tetracycline salts occurs when the material contains moisture. However, the normal moisture content of bulk tetracycline salts should not significantly affect the stability at normal temperatures of storage. The dried phosphate and hydrochloride salts of tetracycline are stable at elevated temperatures. Both phosphate and hydrochloride salts of tetracycline degrade at the same rates in acidic solution. This indicates that both salts provide the same tetracycline ion in the acid of the stomach. Thus, no rationale comparable to the greater resistance to acid degradation of penicillin V over G can be proposed for tetracycline phosphate over the hydrochloride.

The validity of the cylinder-plate assay using *B. subtilis* for tetracycline purity is suspect The cylinder-plate assay depends on two parameters: (*a*) rate of diffusion of the antimicrobial agent through the agar to bring it in contact with a greater volume of microorganisms, and (*b*) the minimum inhibitory concentration in the agar media to inhibit microbial growth. It is feasible that a compound derived from the one to be assayed may have less potency but greater diffusibility so that the radius of the clear areas about the dose may be larger for the least potent material. The question as to the specificity of other test organisms is open.

The shift in the ultraviolet spectra of tetracycline after heat degradation in the bulk form or in solution is consistent with what would be expected if it were transformed in part to anhydrotetracycline with aromatization of the C ring. Pertinent spectrophotometric data are given in Table III.

It is apparent that moist tetracycline salts are not completely transformed by heat to a probable anhydrotetracycline. In addition to the appearance of CO_2 on heat degradation that indicates decarboxamidation, it can be estimated on the basis of the increase in absorptivities given in Table III that only 23% of the molecules are aromatized.

On the basis of this observation, anhydrotetracycline and desdimethylaminotetracycline were run in the same cylinder-plate assay as the tetracycline. Anhydrotetracycline and desdimethylaminotetracycline showed twice the activity on weight basis as tetracycline in this assay. Also, a 50-50 mixture of tetracycline and anhydrotetracycline showed twice the activity on a weight basis as tetracycline.

This "case in point" confirms the importance of the axiomatic statement that the specificity of a biological assay of a drug is just as important to evaluate as is its reliability.

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